CUTTING EDGE

Cutting Edge: Modulation of Airway Inflammation by CpG Oligodeoxynucleotides in a Murine Model of Asthma[1]

Joel N. Kline,[2]* Thomas J. Waldschmidt,† Thomas R. Businga,* Jennifer E. Lemish,* Joel V. Weinstock,* Peter S. Thorne,‡ and Arthur M. Krieg§

Asthma has been increasing in industrialized countries. Evidence suggests that asthma is caused by a Th2 immune response to inhaled environmental Ags and that childhood infections protect against this. We have shown that bacterial DNA contains motifs, centered on unmethylated CpG dinucleotides, which induce Th1-type responses. We hypothesized that the Th1 effect of these CpG motifs may oppose the Th2 type allergic response and suggest that this may account for the protective effect of childhood infection against asthma. We examined the effects of CpG-motif oligodeoxynucleotides (CpG ODN) in a murine model of asthma. Airway eosinophilia, Th2 cytokine induction, IgE production, and bronchial hyperreactivity were prevented by coadministration of CpG ODN with the Ag. Significantly, in a previously sensitized mouse, CpG ODN can prevent allergen-induced airway inflammation. These studies suggest that exposure to CpG DNA may protect against asthma. *The Journal of Immunology*, 1998, 160: 2555–2559.

Bacterial, but not vertebrate, DNA causes activation of B cells and NK cells and the secretion of Th1 cytokines (1–3). These effects result from the presence of unmethylated CpG dinucleotides in particular base contexts (1–3) and can be mimicked with synthetic oligodeoxynucleotides (ODN).[3] In general, Th1 cytokines suppress Th2 responses, implicated in the pathogenesis of asthmatic inflammation (4, 5). Therefore, the Th1 response to bacterial DNA is noteworthy in light of the finding that childhood bacterial or mycobacterial infection protects against asthma and other atopic conditions (6, 7). These data support the hypothesis that during childhood, repeated Ag exposures in the presence of CpG DNA may bias immune responses to Th1 and protect against Th2 type responses such as asthma.

Materials and Methods

Murine model of asthma

C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) were sensitized to *Schistosoma mansoni* eggs (5000, i.p.) and challenged with schistosome egg Ag (SEA, 10 μg intranasal) (8). These eggs were purified from the livers of infected hamsters (9). SEA was prepared by homogenization and concentration of eggs (10).

Oligonucleotides

The CpG ODN consisted of 20 bases containing two CpG motifs (TC CATGACGTTCCTGACGTT). The control ODN was identical except that the CpG motifs are rearranged (TCCATGA*GC*TTCCTGA*GTC*T). ODN were produced by Oligos etc. (Wilsonville, OR) in a Good Manufacturing Practice facility and have undetectable levels of LPS. ODN (30 μg) were administered by i.p. injection.

Whole lung lavage

Following euthanasia, the trachea was cannulated and saline washings were collected. The lavages were processed for cell counts, and the supernatants were saved for further analysis.

Histopathologic examination

At the time of sacrifice, lungs were excised, fixed, and stained with hematoxylin and eosin.

Physiology

Airway hyperreactivity was measured by methacholine-induced airflow obstruction. Mice were placed into whole body plethysmographs (Buxco Electronics, Inc., Troy, NY), interfaced with computers using differential pressure transducers. Measurement was made of respiratory rate, tidal volume, and enhanced pause. Airway resistance is expressed as: $P_{enh} = [(T_e/0.3\ T_r) - 1] \times [2P_{ef}/3P_{if}]$, where P_{enh} = enhanced pause, T_e = expiratory time (seconds), T_r = relaxation time (seconds), P_{ef} = peak expiratory flow (milliliters), and P_{if} = peak inspiratory flow (milliliters/second) (11). Increasing doses of methacholine were administered by nebulization (for 150 s), and P_{enh} were calculated over the subsequent 3 min.

Departments of *Medicine, †Pathology, and ‡Preventive Medicine, University of Iowa College of Medicine, Iowa City, IA 52242 and §the Veteran's Affairs Medical Center, Iowa City, IA 52246.

Received for publication November 26, 1997. Accepted for publication January 14, 1998.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Support for this project was obtained from the National Institutes of Health (KO8HL02950, P30ES05605, R29HL59324), the American Lung Association, the Center for Advanced Studies-Spellman Rockefeller, and Qiagen. A.M.K. is supported by a Career Development Award from the Medical Research Service, Department of Veteran's Affairs.

[2] Address correspondence and reprint requests to Dr. Joel N. Kline, Division of Pulmonary Medicine, University of Iowa Hospitals and Clinics, C33GH, Iowa City, IA 52242. E-mail address: joel-kline@uiowa.edu

[3] Abbreviations used in this paper: ODN, oligodeoxynucleotide; SEA, soluble schistosome egg antigen.

0022-1767/98/$02.00

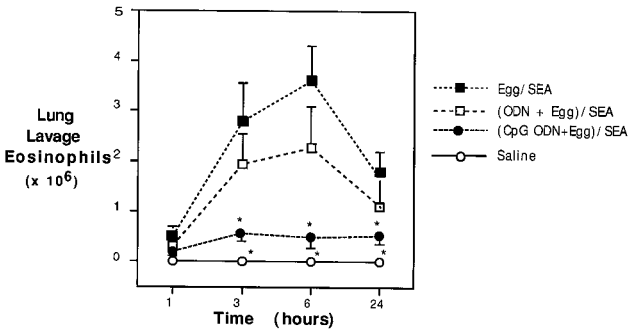

FIGURE 1. CpG ODN prevent airway eosinophilia in a murine model of asthma. C57BL/6 mice were exposed to schistosome eggs with or without ODN (30 μg i.p., day 0) or saline alone. Mice were challenged in the airways with SEA (10 μg) on days 7 and 14. Mice underwent airway lavage at various times following the second challenge. No eosinophils were identified in the bronchoalveolar lavage fluid of control mice at any time point; marked airway eosinophilia was induced by schistosome egg sensitization and challenge and was prevented by coadministration of CpG ODN but not control ODN. Each data point represents the mean ± SEM of at least four individual experiments. *$p < 0.01$ (saline) or ((CpG ODN + egg)/SEA) vs (egg/SEA) or ((ODN + egg)/SEA) mice. There is no significant difference between the (egg/SEA) and the ((ODN + egg)/SEA) mice.

Cytokines

Murine IL-4, IL-12, and IFN-γ were measured using a sandwich ELISA (R&D, Minneapolis, MN). The IL-12 ELISA used a capture Ab specific to the p70 heterodimer.

Measurement of IgE

Total serum IgE was measured using sandwich ELISA. B1E3 (rat IgG anti-murine IgE mAb) was the capture reagent, and biotin-conjugated EM95 (noncompeting rat IgG anti-murine IgE mAb) was used as detection reagent along with alkaline phosphatase-Streptavidin. Affinity-purified monoclonal IgE anti-TNP (A3B1) was used as a standard.

Statistics

Analysis of time course and dose-response curves was performed using ANOVA with post hoc Tukey tests. Pairs of groups were compared using Student's *t* test. *P* values for significance were set at 0.05. Values for all measurements are expressed as the mean ± SEM. Statistical analysis was conducted using Systat 5 for the Macintosh.

Results

To examine the effects of CpG DNA we used a murine model of asthma in which C57BL/6 mice are sensitized to schistosome eggs and challenged with SEA (8). To determine the effect of CpG ODN on the development of airway eosinophilia, we performed lung lavage on mice who received eggs in the presence and absence of CpG ODN or control ODN (each 30 μg i.p., administered at the time of the schistosome eggs) and then were challenged with SEA. Control mice received diluent (saline) alone We found that lung eosinophils (Fig. 1) were significantly greater in the mice exposed to schistosome egg/SEA than in control mice or the group that received CpG ODN along with the schistosome eggs but not greater than in mice that received control ODN. Mice that received CpG ODN also did not develop the peribronchial inflammatory response seen in the egg/SEA mice, whereas those mice that received control ODN with the schistosome eggs were not protected from this inflammation (Fig. 2).

We next evaluated the effect of CpG ODN on bronchial hyperreactivity in this model. With a whole body plethysmograph, mice were monitored after exposure to saline followed by increasing concentrations (12.5–100 mg/ml) of nebulized methacholine. The readout was P_{enh}, which correlates to measured airway resistance (11); values of P_{enh} obtained were normalized to postsaline-P_{enh}, resulting in an index. Mice previously sensitized to schistosome eggs and challenged with SEA developed dose-dependent methacholine-induced bronchospasm that was significantly greater than in control mice or mice that received CpG ODN but no different from that in mice that received control ODN (Fig. 3). These studies confirmed that schistosome Ag-induced bronchial hyperreactivity can be prevented by CpG ODN.

We next examined serum levels of IgE, a marker for atopy. Control mice had 0.66 ± 0.38 μg/ml of IgE, and schistosome egg/SEA mice had significantly greater levels of 4.10 ± 0.54 μg/ml, $p < 0.01$. In contrast, mice that received CpG ODN along with

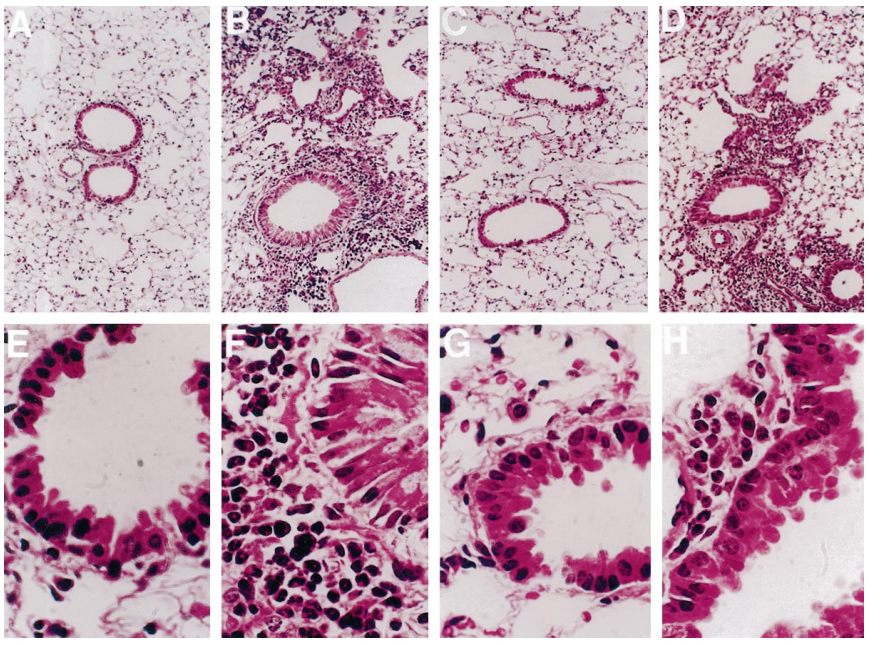

FIGURE 2. CpG ODN prevent peribronchial eosinophilia in a murine model of asthma. C57BL/6 mice were treated as described in Figure 1. These representative sections are representative of at least four individual mice in each group. *A*, *E*, saline control; *B*, *F*, schistosome egg, SEA; *C*, *G*, schistosome egg + CpG ODN, SEA; *D*, *H*, schistosome egg + control ODN, SEA. *A–D*, ×25; *E–H*, ×1000. Significant peribronchial eosinophilic inflammation and epithelial activation, which is induced by SEA in mice sensitized to schistosome eggs, is markedly diminished by coadministration of CpG ODN but not by control ODN.

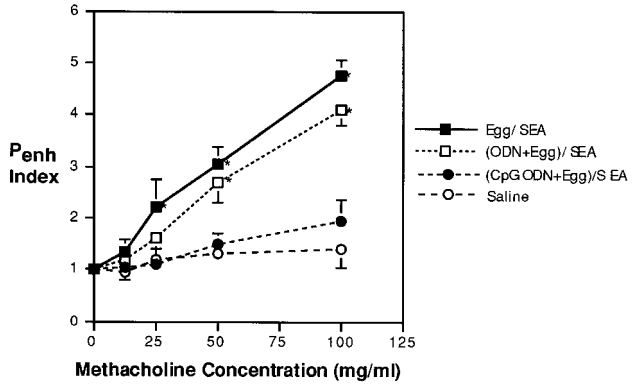

FIGURE 3. CpG ODN inhibit the development of bronchial hyperresponsiveness to inhaled methacholine in a murine model of asthma. C57BL/6 mice were treated as described in Figure 1. Twenty-four hours after the second exposure to SEA, the mice were placed in whole body plethysmographs and underwent methacholine challenge (between 12.5 and 100 mg/ml). The P_{enh} index is a calculated measure of bronchospasm (see *Materials and Methods*). The mice sensitized to schistosome eggs without ODN or in the presence of non-CpG (control) ODN exhibited significant bronchial reactivity to inhaled methacholine, compared with control mice or mice exposed to schistosome eggs in the presence of CpG ODN. $n = 4$ for each group; $*p < 0.05$, vs saline or (ODN + egg)/SEA mice.

the schistosome eggs had an IgE level of 1.04 ± 0.32, which is significantly lower than that of the egg/SEA group ($p < 0.05$) and not different from that of the control group, but mice that received control ODN along with the schistosome eggs had serum IgE levels of 3.04 ± 0.94 μg/ml, which did not significantly differ from the schistosome egg/SEA mice.

CpG ODN also affected lung cytokine release in this model. We first examined the effects of CpG and control ODN alone and found that administration of ODN did not affect lung lavage fluid levels of IL-4, IL-12, or IFN-γ at 1 or at 14 days after administration. IL-4 was significantly increased in egg/SEA-treated mice relative to controls; this was prevented by pretreatment with CpG ODN but with not control ODN (Fig. 4*A*). The IL-4 concentrations in the CpG ODN-treated mice were still elevated above those of control mice. The loss of allergen-induced IL-4 expression in the CpG-treated mice suggests that the Th2 response to allergen exposure was abrogated. We next examined whether CpG treatment would generate an Ag-induced Th1 response. Indeed, we found that both IFN-γ and IL-12, Th1 cytokines, were induced by allergen inhalation in mice primed with the allergen plus CpG ODN (Fig. 4, *B* and *C*); the induction of these cytokines was significant ($p < 0.05$) relative to all other groups. These studies indicate that if an Ag is encountered in the context of CpG DNA, subsequent exposure to the Ag in the lung will lead to a Th1 rather than a Th2 response.

Down-regulation of Ag-driven Th2-mediated responses following sensitization is an important therapeutic goal. To investigate whether CpG DNA may overcome a preexisting Th2 response, we examined the effect of CpG ODN on eosinophilic airway inflammation in mice sensitized to SEA. All mice received schistosome eggs, were reexposed to eggs in the presence of CpG or control ODN or no ODN (day 7), and then were studied following two SEA inhalation challenges (days 14 and 21). Mice given schistosome eggs without ODN developed marked airway eosinophilia ($2.91 \pm 0.70 \times 10^6$ cells). In contrast, the mice that received CpG ODN along with eggs (day 7) developed significantly less airway

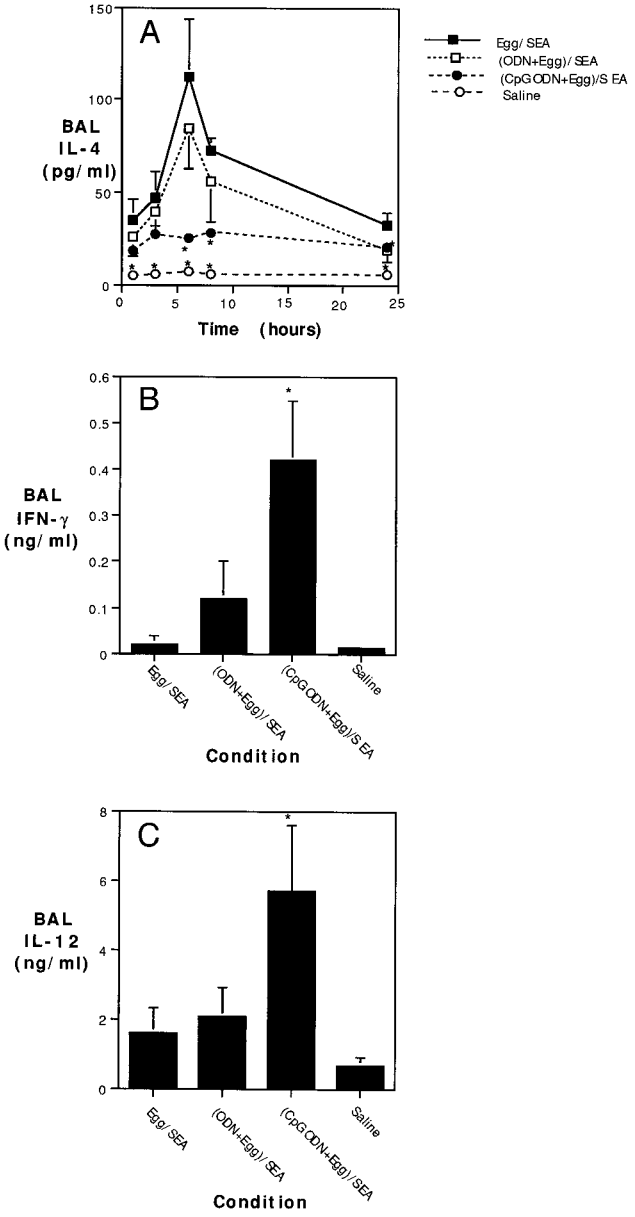

FIGURE 4. CpG ODN diminish the induction of lung IL-4 expression and promote the release of lung IL-12 and IFN-γ in a murine model of asthma. C57BL/6 mice were treated as described above and then sacrificed following lung lavage. *A*, Compared with saline control mice, schistosome egg sensitization primed for the release of IL-4 following SEA challenge which was significantly reduced by coadministration of CpG ODN but not control ODN. $*p < 0.05$ vs egg/SEA mice. CpG ODN, but not control ODN, induced release of IFN-γ (*B*) and IL-12 (*C*) in lavage fluid 6 h after stimulation with SEA, $* p < 0.01$ vs control mice. Each data point represents the mean ± SEM of at least four individual experiments. BAL, bronchoalveolar lavage fluid.

eosinophilia ($0.28 \pm 0.14 \times 10^6$ cells, $p < 0.01$), but the mice that received control ODN and eggs (day 7) developed eosinophilia similar to that of the mice that received schistosome eggs alone (Fig. 5).

These findings demonstrate that the schistosome/SEA model of asthma is characterized by IgE production, airway eosinophilia, pulmonary IL-4 secretion, and bronchial hyperreactivity, which do not develop if CpG ODN are coadministered along with the schistosome eggs. CpG ODN alone do not offer significant protection

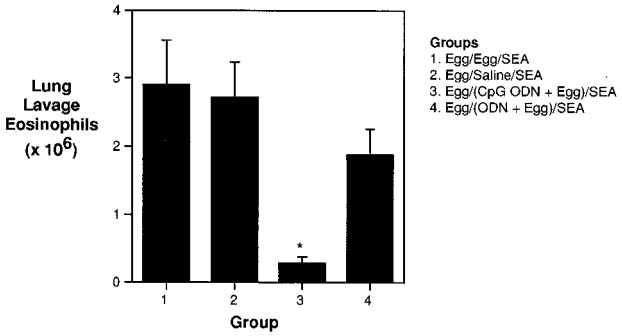

FIGURE 5. Treatment of sensitized mice with CpG ODN and Ag reduces subsequent airway eosinophilia. C57BL/6 mice were sensitized to schistosome eggs (5000 i.p., day 0) and then received saline alone (group 2) or CpG ODN (30 μg i.p.) (group 3) or non-CpG ODN (group 4) along with additional schistosome eggs. All mice then received SEA by intranasal administration (10 μg, days 14 and 21) and were sacrificed 6 h after the final airway challenge. Treatment of schistosome-sensitized mice with CpG ODN and schistosome eggs, but not control ODN and schistosome eggs, decreases subsequent eosinophilic inflammation following exposure to inhaled SEA. Each data point represents the mean ± SEM of at least four individual experiments; * $p < 0.01$ vs group 1.

against the development of airway inflammation. In addition, these effects are Ag specific; in other studies (not shown), CpG ODN can protect against the development of eosinophilic airway inflammation in an OVA murine model of asthma, but protection against OVA sensitization does not confer protection against schistosome sensitization. Both IFN-γ and IL-12 were induced in the mice treated with CpG ODN but not in the mice treated with schistosome eggs and SEA alone, suggesting that induction of Th1-type cytokine expression may be responsible for preventing the eosinophilic airway inflammation. However, the kinetics of expression of these two Th1 cytokines are quite different—plasma IFN-γ levels returned to baseline within 24 h whereas IL-12 levels remained persistently elevated for >8 days following a single injection of CpG ODN (data not shown). CpG ODN is also effective in diminishing the eosinophilic response to Ag even when given following initial sensitization.

Discussion

Immune responses to newly encountered Ags may be generally divided into two classes: Th1, which are characterized by the generation of IFN-γ and IL-12; and Th2, which are associated with a release of IL-4 and IL-5 (12). Bacterial infections are generally a strong trigger for Th1 responses. Asthma is thought to result from the inappropriate generation of Th2 responses to environmental Ags, resulting in acute pulmonary inflammation (5, 13).

The commitment to a Th1 or Th2 response is determined by a variety of factors, including the cytokine milieu in which the Ag is initially presented to specific lymphocytes. If an Ag is initially encountered with Th1 cytokines, then an Ag-driven Th1 response will likely develop. Recent epidemiologic studies suggest that childhood mycobacterial infections protect against later development of atopic conditions, including asthma (6). The incidence of asthma has been rising in industrialized countries in parallel with a decline in the incidence of childhood bacterial and mycobacterial infections (7). These data suggest the possibility that these infections induce a Th1 response and that in their absence, a default to a Th2 response may develop. In societies where childhood infection rates are low, a predisposition to Th2 responses may cause environmental allergies.

We demonstrate here that systemic administration of CpG DNA causes a Th1 rather than a Th2 immune response to schistosome eggs. This raises the possibility that childhood exposure to CpG DNA may restore a Th1 immune influence and reduce the incidence of asthma. Our studies also have implications for treatment of patients previously sensitized to allergens. Current immunotherapy protocols for asthma have little therapeutic effect (14), although immunotherapy can slightly reduce symptoms in selected patients with atopic conditions (15). The beneficial effects of immunotherapy are thought to be at least partly due to induction of Th1 cytokines (16). Our data demonstrating the prevention of eosinophilic airway inflammation in animals already sensitized to schistosome eggs suggest that the potent Th1-like effects of CpG ODN may promote immune desensitization to known allergens. The use of CpG ODN as an adjuvant may dramatically improve the utility of immunotherapy in asthma.

In some models of atopic disease, IL-12 administration can also lead to reduced IL-4 and increased IFN-γ in airway fluid with resultant improvements in eosinophilia (17). However, the use of ODN to protect against eosinophilic inflammation or atopic disease carries several advantages over IL-12 administration. Clinical trials of IL-12 have been associated with substantial morbidity and even mortality (18). Moreover, in some animal models, IL-12 administration can actually worsen eosinophilic inflammation (19). Furthermore, the Th1-promoting effect of IL-12 may be insufficient to suppress a Th2 recall response (20), whereas CpG ODN can prevent airway eosinophilia even after sensitization. The apparent superior Th1 effect of CpG ODN may be due to the fact that it triggers the sustained endogenous production of IL-12 for at least 8 days, while exogenous IL-12 has a relatively short half-life. Finally, oligonucleotides are cheaper to formulate and far more stable than cytokines. CpG ODN may be preferable to the therapeutic use of cytokines, such as IL-12, for reasons of cost, stability, safety, and prolonged expression of induced cytokines. These current studies support the hypothesis that induction of Th1 cytokines by CpG ODN protects against eosinophilic inflammation in asthma, and they suggest that CpG ODN may be an effective novel method of inducing protection against atopic disorders.

Acknowledgments

We thank Laurie Love-Homan, Lorraine Tygrett, Arthur Blum, Marsha O'Neil, and Janine Dekoster for outstanding technical assistance and Erwin Gelfand and Eckhart Hammelmann for assistance in carrying out the airway physiology studies.

References

1. Krieg, A. M., A. K. Yi, S. Matson, T. J. Waldschmidt, G. A. Bishop, R. Teasdale, G. A. Koretzky, and D. M. Klinman. 1995. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature 374:546.*
2. Cowdery, J. S., J. H. Chace, A. K. Yi, and A. M. Krieg. 1996. Bacterial DNA induces NK cells to produce IFN-gamma in vivo and increases the toxicity of lipopolysaccharides. *J. Immunol. 156:4570.*
3. Ballas, Z. K., W. L. Rasmussen, and A. M. Krieg. 1996. Induction of natural killer activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. *J. Immunol. 157:1840.*
4. Corrigan, C. J., A. Haczku, V. Gemou-Engesaeth, S. Doi, Y. Kikuchi, K. Takatsu, S. R. Durham, and A. B. Kay. 1993. CD4 T-lymphocyte activation in asthma is accompanied by increased serum concentrations of interleukin 5: effect of glucocorticoid therapy. *Am. Rev. Respir. Dis. 147:540.*
5. Robinson, D. S., Q. Hamid, S. Ying, A. Tsicopoulos, J. Barkans, A. M. Bentley, C. Corrigan, S. R. Durham, and A. B. Kay. 1992. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N. Engl. J. Med. 326:298.*
6. Shirakawa, T., T. Enomoto, S. Shimazu, and J. M. Hopkin. 1997. The inverse association between tuberculin responses and atopic disorder. *Science 275:77.*
7. von Mutius, E., C. Fritzsch, S. K. Weiland, G. Roll, and H. Magnussen. 1992. Prevalence of asthma and allergic disorders among children in united Germany: a descriptive comparison. *Br. Med. J. 305:1395.*

8. Lukacs, N. W., R. M. Strieter, S. W. Chensue, and S. L. Kunkel. 1994. Interleukin-4-dependent pulmonary eosinophil infiltration in a murine model of asthma. *Am. J. Respir. Cell Mol. Biol. 10:526.*
9. Dresden, M. H., and D. C. Payne. 1981. A sieving method for the collection of schistosome eggs from mouse intestines. *J. Parasitol. 67:450.*
10. Carter, C. E., and D. G. Colley. 1978. An electrophoretic analysis of *Schistosoma mansoni* egg antigen preparation. *J. Parasitol. 64:385.*
11. Hamelmann, E., J. Schwarze, K. Takeda, A. Oshiba, G. L. Larsen, C. G. Irvin, and E. W. Gelfand. 1997. Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. *Am J. Respir. Crit. Care Med. 156:766.*
12. Mosmann, T. R., H. Cherwinski, M. W. Bond, M. A. Giedlin, and R. L. Coffman. 1986. Two types of murine helper T cell clones. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol. 136:2348.*
13. Marini, M., E. Avoni, J. Hollemborg, and S. Nattoli. 1992. Cytokine mRNA profile and cell activation in bronchoalveolar lavage fluid from nonatopic patients with symptomatic asthma. *Chest 102:661.*
14. Norman, P. S., and P. J. Barnes. 1996. Is there a role for immunotherapy in the treatment of asthma? *Am. J. Respir. Crit. Care Med. 154:1225.*
15. Abramson, M. J., R. M. Puy, and J. M. Weiner. 1995. Is allergen immunotherapy effective in asthma? A meta-analysis of randomized controlled trials. *Am J. Respir. Crit. Care Med. 151:969.*
16. Durham, S. R., V. A. Varney, Y. Sun, M. R. Jacobson, R. M. Sudderick, I. S. Mackay, A. B. Kay, and Q. Hamid. 1994. Effect of grass pollen immunotherapy on cell infiltration and cytokine mRNA expression during allergen-induced late nasal response. *J. Allergy Clin. Immunol. 93:230.*
17. Gavett, S. H., D. J. O'Hearn, X. Li, S. K. Huang, F. D. Finkelman, and M. Wills-Karp. 1995. Interleukin 12 inhibits antigen-induced airway hyperresponsiveness, inflammation, and Th2 cytokine expression in mice. *J. Exp. Med. 182:1527.*
18. Marshall, E. 1995. Cancer trial of interleukin-12 halted. *Science 268:1555.*
19. Wynn, T. A., A. Reynolds, S. James, A. W. Cheever, P. Caspar, S. Hieny, D. Jankovic, M. Strand, and A. Sher. 1996. IL-12 enhances vaccine-induced immunity to schistosomes by augmenting both humoral and cell-mediated immune responses against the parasite. *J. Immunol. 157:4068.*
20. Bliss, J., V. Van Cleave, K. Murray, A. Wiencis, M. Ketchum, R. Maylor, T. Haire, C. Resmini, A. K. Abbas, and S. F. Wolf. 1996. IL-12, as an adjuvant, promotes a T helper 1 cell, but does not suppress a T helper 2 cell recall response. *J. Immunol. 156:887.*